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Mutations in Conserved Domain II of the Large (L) Subunit of the Sendai Virus RNA Polymerase Abolish RNA Synthesis

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The large (L) protein of Sendai virus complexes with the phosphoprotein (P) to form the active RNA-dependent RNA polymerase. The L protein is believed to be responsible for all of the catalytic activities of the polymerase associated with transcription and replication. Sequence alignment of the L proteins of negative-strand RNA viruses has revealed six conserved domains (I–VI) thought to be responsible for the enzymatic activities. Charged-to-alanine mutagenesis was carried out in a highly charged, conserved region (amino acids 533–569) within domain II to test the hypothesis of Müller *et al.* [*J. Gen. Virol.* 75, 1345–1352 (1994)] that this region may contribute to the template binding domain of the viral RNA polymerase. The mutant proteins were tested for expression and stability, the ability to synthesize viral RNA *in vitro* and *in vivo*, and protein–protein interactions. Five of the seven mutants were completely defective in all viral RNA synthesis, whereas two mutants showed significant levels of both mRNA and leader RNA synthesis. One of the transcriptionally active mutants also gave genome replication *in vitro* although not *in vivo*. The other mutant was defective in all the replication assays and thus the mutation uncoupled transcription and replication. Because the completely inactive L mutants can bind to the P protein to form the polymerase complex and the polymerases bind to the viral nucleocapsid template, these amino acids are essential for the activity of the L protein.

INTRODUCTION

Sendai virus, a member of the paramyxovirus family, contains a single-stranded, negative-strand genome RNA 15248 nucleotides (nt) in length (for a review, see Lamb and Kolakofsky, 1996). The RNA genome is encapsidated with the nucleocapsid (Nuc) protein (NP) with one NP molecule for every 6 nt (Egelman *et al.,* 1989; Calain and Roux, 1993), and this tight association of RNA and NP in the Nuc renders the RNA nuclease resistant. The viral RNA-dependent RNA polymerase, composed of the phosphoprotein [P, 568 amino acids (aa)] and large (L, 2228 aa) subunits, is packaged in association with the Nuc into virus particles. The RNA polymerases of negative-strand RNA viruses are unique in that they recognize as a template only the encapsidated genome, and not naked RNA, for RNA synthesis. The reproduction of the virus involves the sequential transcription of the Nuc RNA from the precise 3′ end into positive-strand leader (*le*+) RNA and the monocistronic mRNAs, with the gene order of 3′-*le*+-NP-P/V/C-M-F-HN-L-5′. Replicative events produce a full-length complementary (+) sense antigenome RNA encapsidated with NP that serves as the template for the production of progeny encapsidated genomic negative-strand RNA. The viral copy-back defective interfering (DI) particle RNA contains the 5′-terminus of the wild-type genome with just a portion of the L gene and a 3′ end that is complementary to the 5′ end. The DI-H genome used in these studies is 1410 nt in length and has 110 nt of terminal complementarity (Calain *et al.,* 1992).

In vitro studies have shown that the P and L RNA polymerase subunits must be coexpressed in the same cell to form a functional polymerase (Horikami *et al.,* 1992) because L protein is unstable in the absence of P (Horikami *et al.,* 1997). The L protein alone is unable to bind to Nucs, and binding of the polymerase complex occurs through a P–Nuc interaction (Ryan and Portner, 1990; Horikami and Moyer, 1995). The L protein is believed to contain the catalytic activities associated with the polymerase (Einberger *et al.,* 1990; Hammond and Lesnaw, 1987; Hammond *et al.,* 1992; Hercyk *et al.,* 1988; Hunt and Hutchinson, 1993; Poch *et al.,* 1990).

Sequence comparisons of the L proteins of a number of paramyxoviruses and rhabdoviruses revealed six conserved domains, I–VI (Poch *et al.,* 1990; Sidhu *et al.,* 1993). Domain I lies in the amino-terminal end of the protein (aa 225–416) and has no homology to currently known functional motifs. The focus of this study, domain II (aa 503–607), just distal to domain I, may be involved in template recognition. Domain III (aa 653–876) contains the putative polymerase active site. The conserved peptide sequence GDN in this region is similar to the conserved catalytic domain, GDD, of positive-strand RNA virus RNA polymerases (Jablonski *et al.,* 1991). Domains

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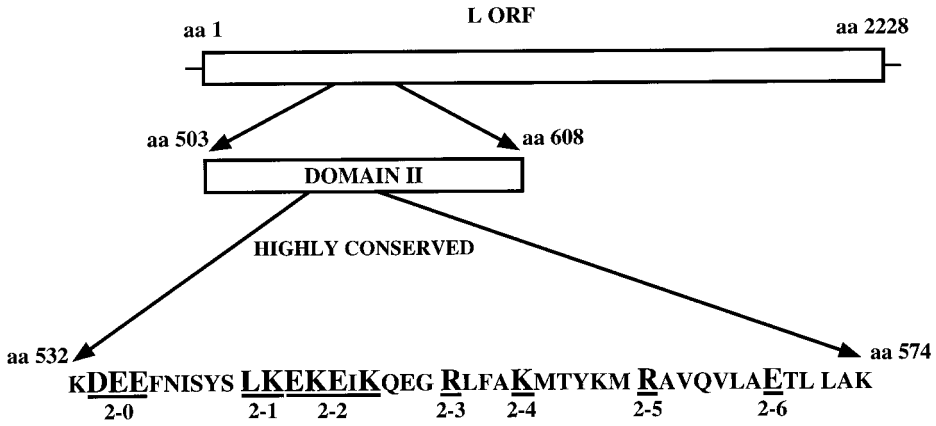
A



B

L PROTEIN	TRANSCRIPTION mRNA	TRANSCRIPTION +leader	REPLICATION in vitro	REPLICATION in vivo
WT L	100	100	100	100
2-0	115	40	26	0
2-1	0	0	0	0
2-2	0	0	0	0
2-3	55	24	0	0
2-4	0	0	0	0
2-5	0	0	0	0
2-6	0	0	0	0

FIG. 1. (A) Schematic representation of the location of the domain II L mutants. The bold, underlined aa were changed to alanine in the mutants numbered as indicated below the sequence. (B) Average of transcription and replication data from three experiments relative to WT L in each case, where the samples varied by less than 10%.

IV and V (aa 927-1128 and aa 1129–1378, respectively) have not shown homology with any known motifs; however, a putative nucleotide binding motif lies within domain VI (aa 1770–1847). Although some of these L domains do share homology with previously characterized functional domains in other proteins, none of the functions of the L domains have been determined.

It is the goal of these studies to test the function of domain II in Sendai L through the use of site-directed mutagenesis and functional analysis of the mutant proteins. Domain II has a central highly conserved region that is highly charged (48%) and is predicted to form an α-helical structure (PredictProtein GenEMBL, Heidelberg). It has been proposed that this region may function in template recognition (Poch *et al.,* 1990). A comparison of the amino acid sequences of 23 different RNA-dependent RNA and DNA polymerases, including human immunodeficiency virus (HIV) reverse transcriptase, showed that there were several regions of extensive homology with similar spacing (Müller *et al.,* 1994) coincident with parts of domains II–IV of the L proteins. The aa within domain II of Sendai L, designated preMotif A (Fig. 1), which were highly conserved or invariant among the representative proteins, including all the L proteins, were targeted for site-directed mutagenesis. With reference to the crystal structure of reverse transcriptase (Jacobo-Molina *et al.,* 1992; Kohlstaedt *et al.,* 1992), Müller *et al.* (1994) proposed that the invariant aa may be in the finger and palm regions of the polymerase that positions the template in the active site of the polypeptide. To decrease possible pleiotropic effects on the structure of the mutants, a charged-to-alanine mutagenesis strategy was used (Diamond and Kirkegaard, 1994; Hassett and Condit, 1994; Ohya and Botstein, 1994; Wiskerchen and Muesing, 1995). By changing charged aa to alanine, it is hoped to disrupt function with minimal disruption of the overall structure of the protein.

RESULTS

The mutant L proteins are expressed *in vivo*

Charged-to-alanine mutations were constructed in highly conserved aa in domain II of the Sendai L protein as shown in Fig. 1A. Because we have an anti-L antibody that will immunoprecipitate L but will not recognize L in an immunoblot, we tested whether the mutant L proteins were expressed by radiolabeling. Cells infected with VVT7 were transfected with the wild-type (WT) or mutant L plasmids together with the P plasmid and incubated with Trans^{35}S label overnight. We have previously shown that coexpression of P with L is necessary for the stable expression of the L protein (Horikami *et al.,* 1997). Immunoprecipitation of extracts of these cells showed that mutants 2-2, 2-4, and 2-6 were expressed at levels com-

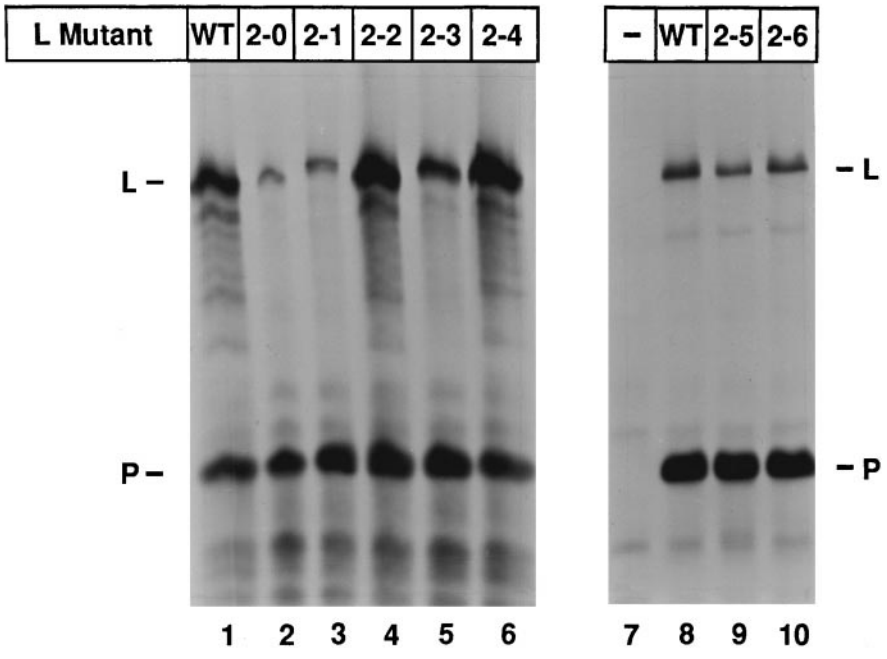


FIG. 2. The mutant L proteins are expressed *in vivo*. A549 cells were infected with VVT7 and transfected with no plasmid (−) or P plasmid together with the indicated WT or mutant L plasmids. The cells were incubated overnight with Trans^{35}S-label, and cytoplasmic cell extracts were prepared. Samples were immunoprecipitated with α-SV and α-L antibodies, and the proteins were analyzed by SDS–PAGE as described in Materials and Methods. The positions of L and P proteins are indicated.

parable to or better than WT L, whereas 2-3 and 2-5 were synthesized at ~50% of the level of WT L (Fig. 2). The expression of mutants 2-0 and 2-1, however, was significantly reduced (80–90%, in multiple experiments), even in a 1-h pulse (data not shown). These data show that all the mutant L proteins were expressed, although at significantly different levels.

Mutations in domain II of L affect mRNA and *le*+ RNA synthesis *in vitro*

To test the effect of the amino acid changes in domain II on the activity of the L protein, VVT7-infected cells were transfected with the P and WT or mutant L plasmids at concentrations optimal for *in vitro* transcription. Cytoplasmic extracts were prepared and incubated with polymerase-free WT RNA-NP template and [α-^{32}P]CTP, and the total mRNA products were analyzed as described in Materials and Methods. Only transcription occurs under these conditions because the NP protein that is required for replication is absent. The WT L and P proteins were active in mRNA synthesis (Fig. 3, lane 2), where the major band contains both the NP and P mRNAs, which comigrate on this gel. In the absence of viral proteins in a sample that was VVT7 infected, but not transfected, there was no transcription of full-length mRNAs, although there were some shorter products that are probably due to residual RNA synthesis by the T7 or vaccinia polymerases (lane 1) that are also present to varying degrees in the other lanes. The mutants 2-0 and 2-3 gave 115% and 55%, respectively, of the activity of WT L, whereas the remainder of the mutants were completely inactive in transcription (Figs. 3 and 1B). The interesting result is that the mutations in 2-0 actually stimulated the activity of the protein by 5- to 10-fold because activity

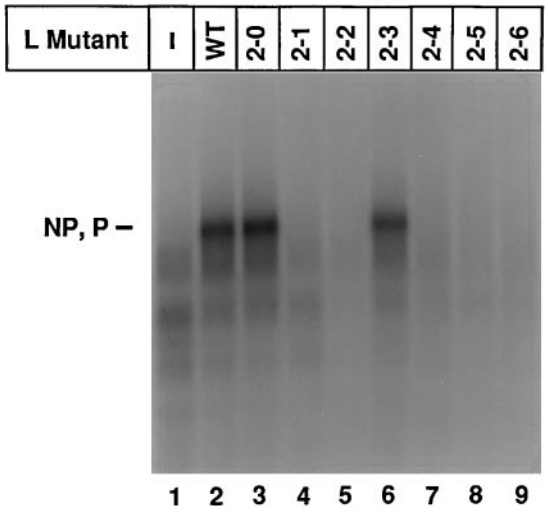


FIG. 3. *In vitro* transcription with the mutant L proteins. A549 cells were infected with VVT7 and transfected with no plasmids (−) or the P plasmid together with the indicated WT or mutant L plasmid. Cytoplasmic cell extracts were prepared and incubated with polymerase-free WT Sendai RNA-NP in the presence of [α-^{32}P]CTP. The labeled RNA was purified and analyzed by agarose–urea gel electrophoresis as described in Materials and Methods. The position of the comigrating NP and P mRNAs is indicated.

was at WT levels but the steady-state level of the 2-0 protein was significantly reduced (80–90%, Fig. 2). The specific activity of mutant 2-3 was about the WT level when corrected for the reduced steady-state amount of protein. Western blot analysis showed that the P protein was equally expressed with each of the mutant L proteins (data not shown). Because charged-to-alanine substitutions in several studies give rise to temperature-sensitive mutants (Diamond and Kirkegaard, 1994, Hasset and Condit, 1994, Ohya and Botstein, 1994, Wiskerchen and Muesing, 1995), we tested for the activity of the domain II L mutants in transcription at 32°C. The same results for mRNA synthesis for each mutant were observed at both 32°C and 37°C, so none were temperature sensitive (data not shown).

Because many of the L mutants were completely inactive in transcription of full-length mRNAs, we also tested whether these proteins were even able to initiate and synthesize the first product of transcription, the *le*+ RNA. Extracts of infected, P and L transfected cells were incubated with the WT RNA-NP template, and the product RNA was detected by Northern blot analysis with a probe specific for *le*+ as described in Materials and Methods. The WT polymerase synthesized *le*+ and, as was previously reported (Vidal and Kolakofsky, 1989; Chandrika *et al.,* 1995), also read through the leader-NP gene boundary to synthesize a spectrum of longer RNAs compared with the negative control without plasmids (Fig. 4, lanes 2 and 1). The mutant L proteins that were inactive in mRNA synthesis also gave no *le*+ synthesis (Fig. 4). However, 2-0 and 2-3 reproducibly synthesized *le*+ RNA at 40% and 24%, respectively, of WT L levels (Fig. 1B), about half the amount of mRNA synthesized by each mutant. These data show that the inactive L mutants cannot even initiate transcription.

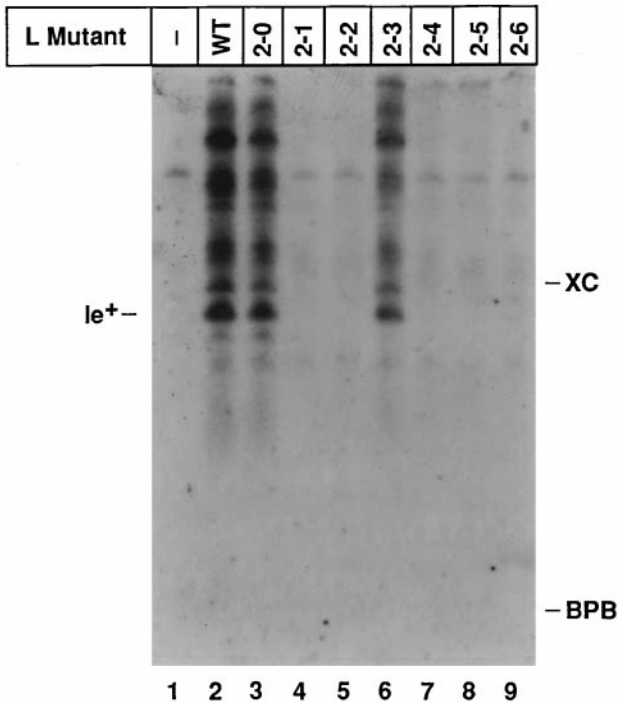


FIG. 4. Synthesis of *le*+ RNA with the L mutants. A549 cells were infected with VVT7 and transfected with no plasmids (–) or the P plasmid cotransfected with the indicated WT or mutant L plasmids. Cytoplasmic cell extracts were prepared and incubated with polymerase-free WT Sendai RNA-NP. The total RNA was extracted, separated on an acrylamide-urea gel, blotted to nitrocellulose, and hybridized with a deoxyoligonucleotide complementary to *le*+ as described in Materials and Methods. The positions of *le*+ RNA and the XC and BPB dye markers are indicated.

Inhibition of replication with mutations in domain II of L

We next tested whether the L mutants were competent for replication of DI-H genome RNA. The *in vitro* replication assay measures the ability of the polymerase to carry out one round of genome synthesis and encapsidation from purified DI RNA-NP template because the amount of product template that could be further replicated is low compared with the amount of added template. Infected cells were transfected with the NP, P, and WT or mutant L plasmids. Cytoplasmic extracts were incubated with DI template and radiolabeled substrate, and the nuclease-resistant, Nuc-associated products were analyzed as described in Materials and Methods. WT L supported good RNA replication, whereas there was no DI-H RNA synthesis in the absence of viral proteins (Fig. 5A, lanes 2 and 1, respectively). The mutant L proteins that showed no transcription activity (2-1, 2-2, 2-4, 2-5, and 2-6) were completely inactive in DI RNA replication as well (Fig. 5A). Mutant 2-0, which could transcribe both mRNA and *le*+ (Figs. 3 and 4), gave some replication of DI-H RNA (Fig. 5, lanes 3, 26%, Fig. 1B), which was, on a molar basis, even more active than WT L. In contrast, 2-3 gave no DI RNA replication (lane 6), although the mutant synthesized significant amounts of mRNA. Western blot analysis showed that the NP and P proteins were equally expressed with each of the mutant L proteins (data not shown).

Another assay for viral genome replication is done *in vivo* where infected cells are transfected with the NP, P, and L plasmids along with a plasmid containing the DI-H gene. In this case, T7 RNA polymerase provides the initial positive-strand transcript of the DI genome RNA that is nonspecifically encapsidated with NP. The viral RNA polymerase then replicates this Nuc-associated DI RNA. This assay requires multiple rounds of replication and encapsidation of both the positive- and negative-strand DI genome RNA to detect product. Nuclease-resistant, Nuc-associated products were isolated and analyzed by Northern blot with a riboprobe specific for negative-strand DI RNA. Although WT L was able to replicate DI RNA *in vivo*, none of the L mutants showed any replication (Fig. 5B), including 2-0, which was able to

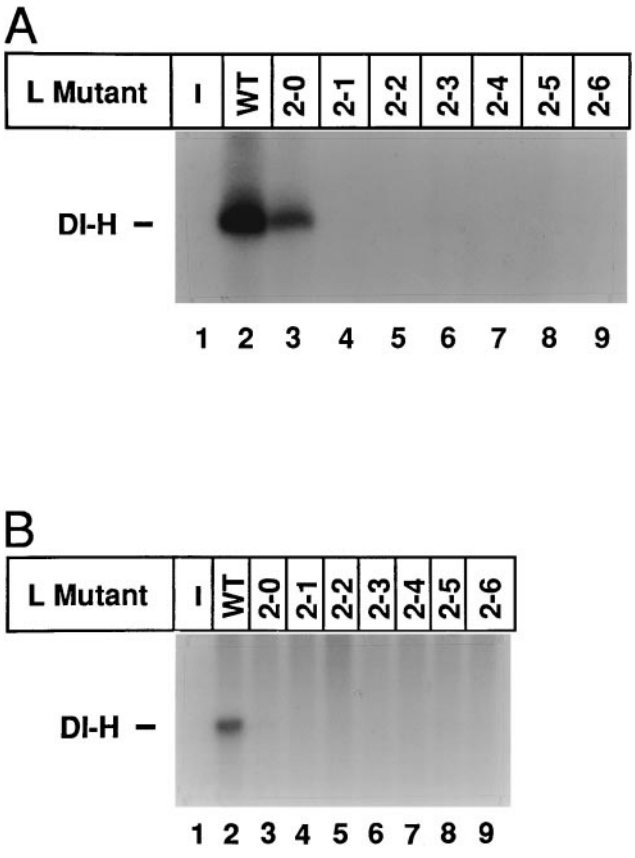


FIG. 5. Replication of DI-H genome RNA with the mutant L proteins. A549 cells were infected with VVT7 and transfected with (A) no plasmids (−) or the P and NP plasmids together with the indicated WT or mutant L plasmid or (B) with pSPDI-H in the absence of the NP, P, and L plasmids (−) or the P, NP, and DI-H plasmids with the WT or mutant L plasmids. Cytoplasmic cell extracts were prepared and incubated (A) in the presence of polymerase-free DI-H RNA-NP in the presence of [α-^{32}P]CTP or (B) not incubated. The samples were nuclease treated, and the purified Nuc RNA was analyzed by agarose-urea gel electrophoresis. (B). Northern blot analysis of *in vivo* replication with ^{32}P-labeled positive-strand DI-H RNA probe. The position of DI-H RNA is indicated.

replicate *in vitro* (Fig. 5A). Thus, although the majority of the alanine substitution mutations in domain II were inactive in replication, two showed differential synthesis of various viral RNAs.

The mutant polymerases bind to the Nuc template

The complete absence of any RNA synthesis in the domain II L mutants 2-1, 2-2, 2-4, 2-5, and 2-6 could be due either to an inhibition of the catalytic activity of the protein or to the loss of essential protein–protein interactions. To initiate RNA synthesis, the P–L complex must form and the polymerase must bind to the template through the interaction of the P, but not L, subunit with Nuc (Ryan and Portner, 1990; Horikami and Moyer, 1995). Thus the Nuc binding assay based on the ability of the polymerase to remain associated with the template during sedimentation simultaneously measures P–L complex formation and binding to the template. Samples of the ^{35}S-labeled cell extracts expressing P and the WT or mutant L proteins in Fig. 2 were incubated in the absence and presence of WT Nuc and subjected to centrifugation through glycerol. Analysis of the pelleted proteins showed that in each case, little or no P and L protein sedimented in the absence of Nuc (Fig. 6, odd-numbered lanes). In the presence of Nuc, however, the same fraction of both the P protein and each of the L mutants bound to the template (Fig. 6, even-numbered lanes) in proportion to the amount of protein synthesized (Fig. 2). Thus each of the inactive mutants formed a P–L complex that bound to the template but did not initiate RNA synthesis.

DISCUSSION

There are six conserved domains, I–VI, that have been defined based on amino acid comparisons of the L proteins of a number of paramyxoviruses and rhabdoviruses (Poch *et al.,* 1990; Sidhu *et al.,* 1993); however, studies directed at identifying specific functions for these regions have been limited. Although the P protein of the P–L polymerase complex brings the enzyme to the template through interaction of the P subunit with NP of the Nuc, the L protein must also interact with the template for RNA synthesis to take place. Domains II and III of L have been proposed to be the polymerase module (Müller *et al.,* 1994). Domain II contains a region, designated pre-Motif A, with two invariant and two highly conserved (>18/23 proteins) charged residues when comparisons are made with 23 RNA-dependent RNA or DNA polymerases, including enzymes from paramyxoviruses, rhabdoviruses, bunyaviruses, arenaviruses, influenza viruses, a filovirus, and HIV (Müller *et al.,* 1994). By extrapolation to the crystal structure of HIV reverse transcriptase, two of these residues, R562 (2-5) and E569 (2-6), are proposed to contribute to the palm region, and two, K453 (2-1) and R552 (2-3), are proposed to contribute to the finger region of the Sendai L protein to position the template in the catalytic site. By site-directed mutagenesis, we changed each of these highly conserved residues to alanine. We also changed other charged residues in this region in 2-0 (D533A, E534A, E535A), 2-2 (E544A, K545A, E546A, K548A), and 2-4 (K556A) that were conserved just in paramyxoviruses and/or rhabdoviruses. With the exception of the 2-2, 2-4, and 2-6 proteins, which were synthesized at or near WT L levels, the remainder of the changes reduced the steady-state levels of the mutant proteins, particularly for 2-0 and 2-1, which were only 10–20% of WT L. Thus some of the alanine substitutions for the charged residues had significant effects on the overall stability of the protein.

As summarized in Fig. 1B, mutants 2-1, 2-2, 2-4, 2-5, and 2-6 are completely inactive in all viral RNA synthesis. Although these aa are thus essential for the activity of L

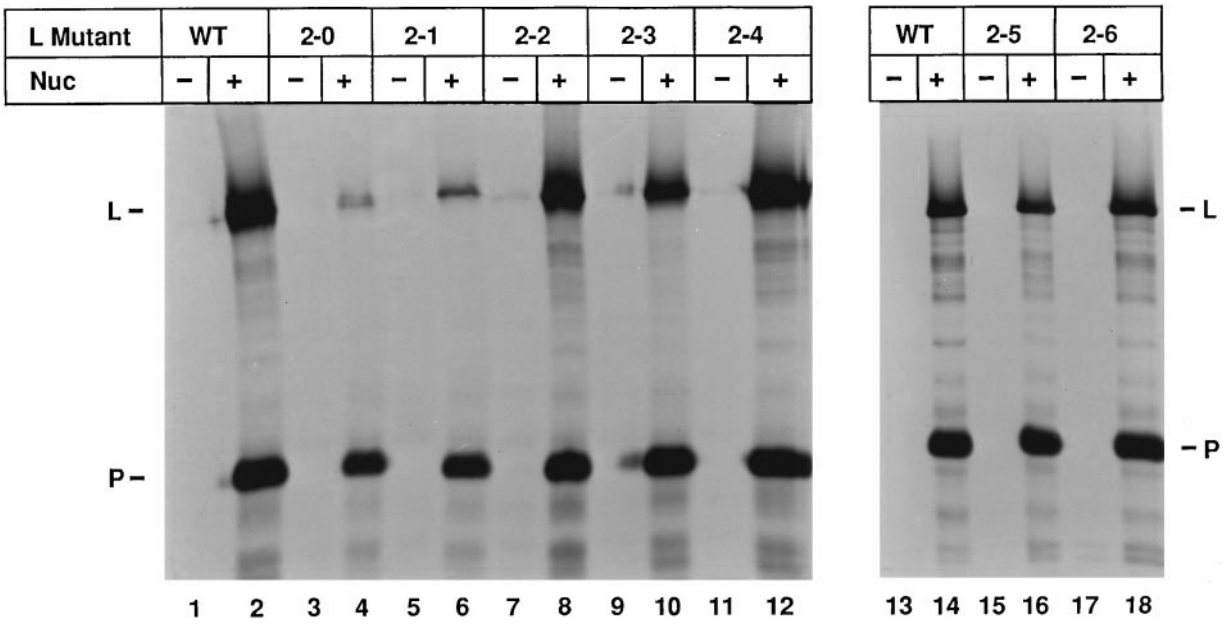


FIG. 6. Polymerase complexes formed with mutant L proteins bind Nucs. Samples of the ^{35}S-labeled cell extracts expressing the P and WT or mutant L proteins from Fig. 2 were incubated in the absence (−) or presence (+) of Sendai polymerase-free RNA-NP (Nuc). The Nucs were pelleted through glycerol, and the Nuc-associated proteins were analyzed by SDS–PAGE as described in Materials and Methods. The positions of L and P proteins are indicated.

protein, as might have been expected from their conservation, the results do not allow the determination of their specific function in RNA synthesis. Surprisingly, mutant 2-0 with changes that are conserved only in paramyxoviruses and rhabdoviruses gave as much mRNA synthesis as WT L with much reduced levels of protein. Thus on a molar basis, 2-0 is actually 5- to 10-fold more active than WT L. In contrast, mutant 2-0 gave 26% and 0% of WT L in *in vitro* and *in vivo* replication, respectively. Thus replication *in vitro* is impaired relative to transcription. Replication *in vitro* measures just a single round of synthesis and encapsidation from an added template, whereas *in vivo* replication requires multiple rounds to see Nuc-associated genomic product. Apparently, the reduced replicative activity of 2-0, as well as the reduced steady-state levels of the protein, prevented detectable levels of genome synthesis within transfected cells.

Mutant 2-3 with the change R552A, which is invariant in 23 polymerases, interestingly gave activity in transcription but was defective in all genome replication, thus uncoupling the two processes. It appears that R552, which is postulated to be in the finger region, is not involved in catalytic activity, a defect that might have been expected to abolish all RNA synthesis as we observed for the majority of the mutations in domain II. Because this amino acid is completely conserved, perhaps it has an essential function that has not been tested by the assays used here. Alternatively, the extrapolation of the HIV reverse transcriptase structure to the active site of negative-strand RNA virus polymerases may not be justified, particularly because the template for L protein is a ribonucleoprotein particle rather than naked RNA. We previously identified several other Sendai L mutations in domain I, an insertion at G348 and substitutions at T349/S350 and K354/A355 (Chandrika *et al.,* 1995) and a substitution at C1571 between domains V and VI (Horikami and Moyer, 1995), that gave a similar phenotype where transcription occurred, but replication was completely inhibited, suggesting that different regions of L participate specifically in one or more steps of replication.

Leader RNA synthesis was also observed for both 2-0 and 2-3, which could synthesize mRNAs; however, the level of *le*+ synthesis was about half the level of mRNA synthesis. Normally *le*+ RNA synthesis is similar to mRNA synthesis. There are two possible mechanisms to account for this discrepancy. First, the polymerases may actually initiate in these cases at the NP start signal as well as at the 3′ end at the *le*+ gene, which is not usually the case. There is precedent for one mutation in vesicular stomatitis virus (VSV) N protein to promote initiation directly at the N gene start site (Chuang and Perrault, 1997). Alternatively, the mutant polymerases may now readthrough the *le*+–NP gene boundary synthesizing a fused *le*+–NP transcript, which would reduce the amount of *le*+ RNA. In measles virus-infected cells, some polyadenylated N RNA contains a leader sequence; however, this RNA is encapsidated (Castaneda and Wong, 1990).

Direct investigations of other conserved domains within the L proteins of several negative-strand RNA viruses have yielded interesting results. A putative poly-

TABLE 1

Mutant Sendai Virus L Proteins

Mutant	Mutagenic oligonucleotide[a]	Silent site	Amino acid change[b]
WT*	SM274-GTACACTGAATC**CGTACG**GGGGACGCCTGAG	*Bsi*WI	None
2-0	SM315-CGAGATGTTGAA**AGCTGC**TGCTTTCAACCAATCTCC	*Bso*FI	D533A, E534A, E535A
2-1	SM316-GCTTGATCTCTTTCTCT**GCAGC**ACTGTACGAGATG	*Bso*FI	L542A, K543A
2-2	SM317-GACCCTCTTG**TGC**GAT**TGC**T**GCAGC**TTTGAGACTGTACG	*Bso*FI	E544A, K545A, E546A, K548A
2-3	SM318-GCGAATAG**GGCG**CCCTCTTGCTTG	*Nar*I	R552A
2-4	SM319-CGCATCTTATAAGTCAT**AGCTGC**GAATAGACGAC	*Bso*FI	K556A
2-5	SM320-CACCTGTACG**GCTGC**CATCTTATAAGTC	*Bso*FI	R562A
2-6	SM321-GCCAGTAGTGT**GGCTGC**CAGCAC	*Bso*FI	E569A

[a] Mutant oligonucleotides are of the noncoding sense and listed in the 5′-3′ direction. The underlined sequence indicates the silent restriction enzyme site, and bold letters indicate the nucleotide changes. WT* contains a *Bsi*WI silent site at nt 1828 for cloning.

[b] Amino acid changes are listed by the wild-type amino acid, the position in the protein, and the amino acid change.

merase module in domain III, containing the GDN sequence motif C, was identified. These residues are proposed to lie within the palm region of the RNA polymerase constituting the active site (Müller *et al.,* 1994). Analyses of site-directed mutants of this region of bunyamwera virus L (Jin and Elliott, 1992), VSV L (Sleat and Banerjee, 1992), and rabies virus L (Schnell and Conzelmann, 1995) proteins have shown that GDN is essential for *in vivo* or *in vitro* RNA synthesis because RNA synthesis is lost in the mutants. Mutations made near this putative catalytic core either completely or partially disrupted RNA synthesis. These data show that the conserved aa GDN are strictly required for RNA synthesis but do not address whether their function is actually in the catalytic site of the polymerase.

A role of the VSV L protein in polyadenylation was also shown where a specific mutation at aa 1488 between domains V and VI conferred an aberrant polyadenylation phenotype in which the viral mRNAs possessed extremely long poly(A)$^+$ tails (Hunt and Hutchinson, 1993). A similar study in which multiple amino acid substitutions were made at the corresponding amino acid, C1571, in the Sendai L protein yielded multiple defective RNA synthesis phenotypes, although aberrant poly(A)$^+$ tail length was not among them (Horikami and Moyer, 1995). Studies using host range mutants of VSV provided evidence that specifically linked methyltransferase activities to the L protein subunit of the viral polymerase (Hercyk *et al.,* 1988), although the nature of the mutations is not known.

MATERIALS AND METHODS

Cells, viruses, plasmids, and antibodies

Human lung carcinoma cells (A549) were used for all experiments. WT Sendai virus (Harris strain) and the Sendai virus defective interfering particle, DI-H, were propagated and purified as described previously (Carlsen *et al.,* 1985). Recombinant vaccinia virus containing the gene for T7 RNA polymerase (VVT7) (Fuerst *et al.,* 1986) was grown in Vero cells. The plasmids pGem-L, pGem-NP, and pGem-Pstop (which expresses P, but not the C proteins) were described by Curran *et al.* (1991). The DI-H clone, pSPDI-H, was described by Myers and Moyer (1997). All of the genes were cloned downstream of the phage T7 promoter. Immunoprecipitation used rabbit polyclonal anti-Sendai virus antibody (α-SV) and rabbit polyclonal anti-trpE-L fusion antibody specific for aa 641-1256 of the Sendai L protein (α-L) (Horikami *et al.,* 1992).

Construction of Sendai L mutant plasmids

The Sendai virus L protein mutants were constructed using single-stranded DNA with an oligonucleotide-directed mutagenesis kit (Amersham Sculptor In Vitro Mutagenesis Kit) according to the manufacturer's protocol. To make phagemid DNA, a portion of the L gene encompassing domain II from nt 1140 to 3498 using the *Apa*I and *Sac*I sites was subcloned into pBSKS$^+$ vector (Stratagene) at those sites. The single-stranded DNA was isolated, and a plasmid [pBSKS-*Apa*I(*Bsi*WI)*Sac*I] containing a silent *Bsi*WI restriction site at nt 1828 in the L fragment was constructed by site-directed mutagenesis with the primer WT* shown in Table 1 to allow subcloning of a smaller fragment during the subsequent mutagenesis. The *Apa*I(*Bsi*WI)*Sac*I fragment was sequenced and subcloned back into pGem-L at the *Apa*I and *Sac*I sites to create WT*L. The WT*L protein was identical in activity in RNA synthesis to the original L protein as expected (data not shown) and was used as WT L in all the subsequent experiments. Single-stranded DNA was isolated from the pBSKS-*Apa*I(*Bsi*WI)*Sac*I plasmid and used for site-directed mutagenesis in domain II with the primers shown in Table 1. The mutagenic oligonucleotides were designed to contain a silent restriction site to facilitate screening. The mutant fragments were then subcloned back into the WT*L plasmid using the *Pml*I and *Bsi*WI sites, and the resultant L mutants are

listed in Table 1. The authenticity of each subcloned fragment was confirmed by sequencing by the DNA Sequencing Core of the University of Florida.

Protein analysis

Subconfluent A549 cells (100-mm dishes) were infected with VVT7 at a multiplicity of infection of 2.5 PFU/cell for 1 h at 37°C and transfected with Lipofectin (GIBCO-BRL) with the WT or mutant L plasmids (15 μg) with pGem-P (15 μg). At 5 h post-transfection (p.t.), the cells were incubated with Trans^{35}S-label (75 μCi/ml) at 37°C for 18 h in 0.1× cysteine and methionine containing DMEM. Extracts were prepared at 4°C through lysolecithin permeabilization as described previously (Horikami *et al.,* 1992) in reaction mix salts (0.1 M HEPES, pH 8.5, 0.05 M ammonium chloride, 4.5 mM magnesium acetate) and pelleted at 4°C for 30 min at 13,000 rpm. For immunoprecipitation, samples (40 μl) were incubated with 1 μl each α-SV and α-L antibodies and *Staphylcoccus aureus* (Cowan strain) as described previously (Carlsen *et al.,* 1985), and the bound proteins were analyzed by 7.5% SDS–PAGE and autoradiography. For the Nuc binding assay, samples (125 μl) of the ^{35}S- labeled cytoplasmic cell extracts were incubated without or with polymerase-free WT RNA-NP (Nuc) (1 μg) for 30 min at 37°C. The Nucs were pelleted through a step gradient with 2.5 ml of 30% and 50% (w/v) glycerol in 10 mM HEPES, pH 8.5/1 mM EDTA in the SW 55 rotor at 50,000 rpm for 75 min at 4°C. The pellets were resuspended, and the bound proteins were analyzed by SDS–PAGE and autoradiography.

RNA synthesis

Subconfluent A549 cells (60-mm dishes) were infected with VVT7 at a multiplicity of infection of 2.5 for 1 h at 37°C and transfected for transcription with the WT or mutant L plasmids (0.5 μg) and pGem-P (1.5 μg). For replication *in vitro*, transfection was with the L (0.5 μg), NP (2 μg), and P (5 μg) plasmids and for *in vivo* with these amounts of L, NP, and P plasmids with the addition of pSPDI-H (2.5 μg). At 18 h p.t., cytoplasmic extracts were prepared using lysolecithin permeabilization in reaction mix as described previously (Chandrika *et al.,* 1995). Extracts for transcription were treated with micrococcal nuclease (12 μg/ml) with 1 mM $CaCl_2$ for 30 min at 30°C, and EGTA (2.2 mM) was added. Transcription or replication *in vitro* was carried out for 2 h at 30°C with the addition of [α-^{32}P]CTP (500 μCi/ml, Amersham, Dupont) and polymerase-free WT Sendai virus RNA-NP template (1 μg) or DI-H RNA-NP template (2 μg), respectively, prepared as described previously (Horikami *et al.,* 1992). The total transcription products were purified on a Qiagen RNeasy Total RNA kit according to the manufacturer's protocol. Replication products were incubated with micrococcal nuclease and then EGTA as described above, and the nuclease-resistant, Nuc-associated RNA was isolated with the RNeasy kit. The mRNA and replication RNA products were analyzed by 1.5% agarose acid–6 M urea gel electrophoresis (Carlsen *et al.,* 1985) and autoradiography. *In vivo* negative-strand DI-H replication products were detected by Northern analysis with positive-strand ^{32}P-labeled T7 RNA polymerase transcript from *Xba*I linearized pSPDI-H plasmid. For *le*+ RNA synthesis during transcription, unlabeled product RNA was phenol extracted, separated by electrophoresis on an 8% polyacrylamide–8 M urea gel, and electroblotted onto Hybond-N nitrocellulose. The *le*+ RNA was detected by Northern analysis with a ^{32}P-end-labeled complementary deoxyoligonucleotide. The amount of radiolabel in each of the products was determined on the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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